ARTICLES

HEMAGGLUTININ COMPATIBILITY BETWEEN AVIAN AND HUMAN INFLUENZA A VIRUSES USING HUMAN MATRIX PROTEIN: BASED ON SCHOLTISSEK ET AL.'S (2002) ARTICLE

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ABSTACT

Through the review of Scholtissek et al.[1], evolution between different strains of influenza A viruses were examined to enable better preparation for future pandemics. Pandemics are the result of antigenic shifts, cumulative reassortants between circulating viruses that form novel gene sequences. The process may produce a virus which a large segment of the population has no immunological memory of, and consequently, are susceptible to the strain.

The pandemics in 1918, 1967, 1968 and 2009 were caused by influenza A viruses with hemagglutinin (HA) proteins of 1, 2, or 3 - three out of sixteen known HA subtypes. This raises the question whether pandemics can contain other HA subtypes. Since influenza viruses have segmented genomes, it may require at least two different strains to swap their gene segments in order to co-infect a cell; the better viral compatibility between the parent viruses, the more virulent the reassortant is. A collection of HA subtypes in avian strains and Matrix (M) protein in human strains were used in the experimental model by Scholtissek et al. to examine the recombinants' survivability and virulence. Although the results conclude that it is not possible for future pandemics to contain other HA subtypes, the work of Scholtissek et al. leads to further research on influenza A reservoirs.

Ce document est un résumé au sujet de l'article de Christoph Scholtissek[1] publié en 2002. J'examinerai son modèle expérimental, en mettant en évidence les résultats et donnant un aperçu des recherches plus élaborées. En étudiant des modèles de la coopération entre les virus, ceci permet d'aider à se préparer face aux futures pandémies et épidémies. De tels évènements sont causés par des changements antigéniques produits par l'accumulation de réassortiments entre les virus en circulation et divers éléments. Les virus grippaux A sont en constante évolution, et nécessitent une surveillance constante en anticipation à une pandémie. Les pandémies antérieures, soient celles en 1918, 1957, 1968 et 2009, ont démontré à avoir les hémagglutinines (HA) 1, 2 et 3 – trois des seize sous-types HA possibles. Ceci remet en question la possibilité que les pandémies puissent contenir d'autres sous-types HA. Afin que les virus puissent former des virus réassortis potentiellement nouveaux ils doivent bien coopérer, ce qui est précisément ce que Scholtissek tente d'enquêter. Son modèle expérimental implique des réassortiments entre les différents sous-types d'HA dans des souches aviaires et des souches humaines détenant des M-protéines, afin de déterminer la compatibilité virale. Bien que les résultats concluent qu'il est très peu probable que de futures pandémies détiennent d'autres sous-types HA, ils fournissent des indices du potentiel pandémique. En outre, son article incite la recherche plus à fond sur d'autres réservoirs de la grippe A, les méthodes pour surmonter les barrières entre espèces et le réassortiment efficaces.

INTRODUCTION

When influenza viruses co-infect a cell, genetic recombination occurs as the new virus obtains different traits from both parents. Mutation is important for viruses to be able to replicate efficiently, as it results in resistance to anti-viral drugs like amantadine. Amantadine inhibits M2 ion-channels and its related function in viral replication. In order to assess recombination, two selection tools were used against avian M genes and human HA genes. The first, a control and variable virus was used, one being amantadine-resistant and the latter being amantadine sensitive. The second selection tool was hyperimmune antisera, which was used in all the samples.

Currently, there are 16 known HA subtypes in avian influenza A strains.[2] Given the previous occurrences of 1918 A(H1N1), 1957 A(H2N2) and 1968 A(H3N2),

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it is known that H1, H2, and H3 can be found in influenza A pandemics. HA is an essential component in the virus' genome as it is responsible for viral replication. Its functions include the binding of the cells' sialic acid-containing receptors for infection, and when the virus undergoes fusion.

The M protein of human influenza A viruses, is split up between M1 and M2[1]. The proteins keep the core of the virion and its viral envelope intact while also being responsible in the viral replication cycle as its ion channel permits the uncoating of the virus.[3]

Interactions such as the avian strain recombining with the human strain require sufficient cooperation, where the genes reassort to make a new sequence. Cooperation is determined in the experimental model when a viable reassortant virus contains the avian HA gene and the human M gene, meaning that the HA genes of both parent viruses successfully reassorted. Studying the compatibility between avian HA and human M genes is valuable as it provides clues in pandemic potential.

MATERIALS & METHODS

Preparation Before Conducting the Experiment[(1)]

In order to conduct the experiments, the viruses were prepared and plaque-purified in MDCK cells before being stocked in 10-day embryonated chicken eggs. The amantadine-resistant (Am+) viruses were cultured in the presence of 2μg of amantadine before further plaque-purification. A stock of allantoic fluid was obtained for each virus strain with the desired genetic traits required for the experiment.

Scholtissek Experimental system testing compatibility between Human M-gene and avian HA[(1)] The tables attached shows the MDCK cells either singly or doubly infected. Depending on the set, either 1:100 or 1:200 ratios were used to dilute hyperimmune antisera in PBS. There were three sets of experiments conducted:

1. Two different A/PR/8/34 (H1N1) strains were used in experimenting with most of the HA subtypes in avian influenza viruses (Table 1).
2. Sing/57 (H2N2) was used when doubly infecting the cells with avian influenza viruses. Two samples of A/Swine/Germany/81 were used, one being singly infected, and the other being doubly infected with Sing/57 (Table 2).
3. A/Nanchang/933/95 (H3N2) was used when doubly infecting the cells with avian strains. A/Swine/Germany/81 was used for two samples, similar to the previous set (Table 3).

Each sample was treated with 1μg/ml of TCPK and 2 ml of 4% bovine serum.[1] The viruses were incubated for 20 hours before they were split into two groups - selection and no selection. The "selection" groups were diluted with hyperimmune antisera to isolate against human HA's (αH2). The "no selection" group were used without further treatment (Figure 1).

The infected MDCK cells were treated with 0.9% agar and 4% bovine serum albumin and 0.5 μg of TCPK/ml. The "selection" group had 4μg/ml of amantadine in its agar overlay. After leaving the plaques for 3 days at 37°C, certain plaques were observed and stained with 0.1% crystal violet containing 10% formaldehyde.[1] Plaques that needed further purification were dissolved in 1 ml of PBS, until ready for staining and observation.

RESULTS

(Am+ human PR8 strains) X (Am- avian influenza A viruses)[(1)]

According to Table 1, the recombinants were able to produce many, well-distinct plaques. The results indicated that amantadine-resistant human PR8 strain and amantadine-sensitive avian influenza A viruses were able to reassort strains that replicate well.

(Am+ human Singapore strain) X (Am- avian Influenza A viruses)[(1)]

Most of the reassortments were produced few plaques, and were not viable (Table 2). The HA in the avian-like swine influenza viruses (H1N1) were more successful in cooperating with the HA in Sing/57 than the avian strains.

(Am+ human Nanchang strain) X (Am- avian influenza A viruses)[(1)]

As presented in Table 3, the recombinants were unable to replicate efficiently; the overall performance was worse than the second set with the Singapore 1957(H2N2) strain. Scholtissek et al. tested the human Nanchang strain with two different avian-like swine strains with similarly poor results.

Figure 1: *Experimental design of double infection of MDCK cells and selection of influenza virus reassortants that carry the HA gene of the avain virus and the M gene of the amantadine-resistant variant of the human Singapore influenza virus. The hyperimmune antiserum (αH2) was directed against the HA of the human Singapore virus.*

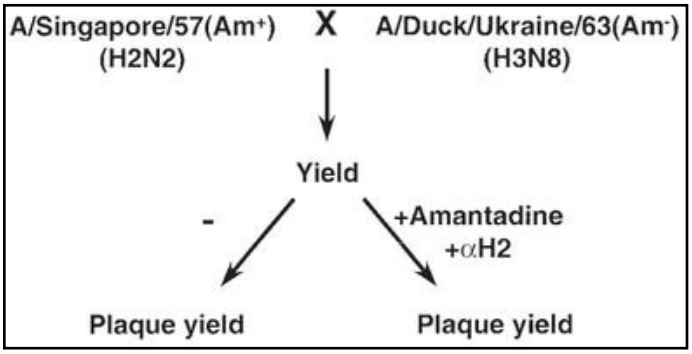

Figure 1. Scholtissek et al.'s (2002) experimental design to test for compatibility between human M-gene and avian HA. From *Cooperation between the Hemagglutinin of Avian Viruses and the Matrix Protein of Human Influenza A Viruses Christoph Scholtissek, Jürgen Stech, Scott Krauss, and Robert G. Webster J. Virol. February 2002 76:1781-1786; doi:10.1128/JVI.76.4.1781-1786.2002*

Table 1: *Plaque yields (PFU) and maximum plaque diameters after a 20-h single or double infection with human PR8 and avian influenza A viruses.*

Virus strain(s)	PFU (maximum plaque diam [mm])	
	No selection	Selection[a]
A/PR/8/34 (H1N1)[b]	2×10^8 (3)	$<10^3$
A/Mallard/Potsdam/178-4/83 (H2N2)	2×10^8 (5)	$<10^4$
A/Mallard/Potsdam/178-4/83, PR8	5×10^7 (5)	6×10^5 (5)
A/Duck/Ukraine/63 (H3N8)	2×10^8 (6)	$<10^4$
A/Duck/Ukraine/63, PR8	8×10^7 (6)	4×10^7 (6)
A/Duck/Hong Kong/Y264/97 (H4N8)	1×10^7 (4)	$<10^3$
A/Duck/Hong Kong/Y264/97, PR8	2×10^7 (3)	2×10^5 (3)
A/Duck/Singapore/3/97 (H5N3)	1×10^8 (2)	$<10^3$
A/Duck/Singapore/3/97, PR8	1×10^8 (3)	6×10^6 (2)
A/Gray Teal/Australia/1/79 (H7N8)	2×10^7 (3)	$<10^3$
A/Gray Teal/Australia/1/79, PR8	2×10^7 (5)	1×10^6 (5)
A/Chick/Germany N/49 (H10N7)	3×10^7 (3)	$<10^3$
A/Chick/Germany N/49, PR8	2×10^7 (4)	3×10^6 (4)
A/Duck/Hong Kong/P50/97 (H11N9)	5×10^7 (2)	$<10^3$
A/Duck/Hong Kong/P50/97, PR8	3×10^7 (2)	4×10^6 (3)
A/Mallard/Astrachan/263/82 (H14N5)	4×10^7 (4)	$<10^3$
A/Mallard/Astrachan/263/82, PR8	4×10^7 (4)	4×10^6 (4)
A/Wedge-tailed Shearwater/Australia/79 (H15/N9)	2×10^8 (4)	$<10^4$
A/Wedge-tailed Shearwater/Australia/79, PR8	5×10^7 (3)	8×10^6 (3)

[a] Anti-HI antiserum (1:100 dilution in PBS) was used to select against human HA, and amantadine (4 μg/ml in the agar overlay) was used to select against avian M genes.

[b] The PR8 virus is naturally amantadine resistant (5).

Table 1. Table from Scholtissek et al. (2002) to illustrate plaque yields and diameters for human strains of PR8 and avian influenza A. From *Cooperation between the Hemagglutinin of Avian Viruses and the Matrix Protein of Human Influenza A Viruses Christoph Scholtissek, Jürgen Stech, Scott Krauss, and Robert G. Webster J. Virol. February 2002 76:1781-1786; doi:10.1128/JVI.76.4.1781-1786.2002*

Table 2: *Plaque yields (PFU) and maximum plaque after single or dougle infection of MDCK cells with the human Singapore and avain or swine influenza A viruses.*

Virus strain(s)	PFU (maximum plaque diam [mm])	
	No selection	Selection[a]
A/Singapore/57 (H2N2)[b]	2×10^8 (2)	$<10^2$
A/Oystercatcher/Germany/87 (H1N1)	4×10^6 (1.5)	$<10^3$
A/Oystercatcher/Germany/87, Singapore	1×10^6 (2)	2×10^2 (0.1)
A/Duck/Alberta/35/76 (H1N1)	4×10^7 (2)	$<10^3$
A/Duck/Alberta/35/76, Singapore	1×10^8 (2)	2×10^4 (0.2)
A/Duck/Ukraine/63 (H3N8)	2×10^8 (6)	$<10^4$
A/Duck/Ukraine/63, Singapore	2×10^8 (6)	1.3×10^8 (6)
A/Duck/Hong Kong/Y264/97 (H4N8)	3×10^7 (4)	$<10^3$
A/Duck/Hong Kong/Y264/97, Singapore	1×10^7 (4)	1×10^6 (5)
A/Duck/Singapore/3/97 (H5N3)	2×10^8 (3)	$<10^3$
A/Duck/Singapore/3/97, Singapore	2×10^8 (3)	2×10^5 (0.5)
A/Chick/Germany N/49 (H10N7)	5×10^7 (2)	$<10^5$
A/Chick/Germany N/49, Singapore	8×10^7 (2)	1.5×10^5 (0.2)
A/Duck/Hong Kong/P50/97 (H11N9)	3×10^8 (3)	$<10^3$
A/Duck/Hong Kong/P50/97, Singapore	2×10^8 (4)	2×10^7 (4)
A/Mallard/Astrachan/263/82 (H14N5)	2×10^7 (4)	$<10^4$
A/Mallard/Astrachan/263/82, Singapore	3×10^7 (4)	5×10^4 (0.4)
A/Wedge-tailed Shearwater/Australia/79 (H15/N9)	8×10^8 (6)	$<10^4$
A/Wedge-tailed Shearwater/Australia/79, Singapore	8×10^7 (4)	5×10^5 (0.4)
A/Swine/Germany/81 (H1N1)	8×10^7 (3)	$<10^4$
A/Swine/Germany/81, Singapore	2×10^7 (3)	2×10^6 (3)

[a] Anti-H2 antiserum (1:200 dilution in PBS) was used to select against human HA, and amantadine (4 μg/ml in the agar overlay) was used to select against avian and swine M genes.

[b] An amantadine-resistant Singapore variant was used.

Table 2. Table from Scholtissek et al. (2002) to illustrate plaque yields and diameters for human Singapore strain and avian influenza A. From *Cooperation between the Hemagglutinin of Avian Viruses and the Matrix Protein of Human Influenza A Viruses Christoph Scholtissek, Jürgen Stech, Scott Krauss, and Robert G. Webster J. Virol. February 2002 76:1781-1786; doi:10.1128/JVI.76.4.1781-1786.2002*

DISCUSSION

If the reassortants produce a great yield in clear plaques, it implies that the parent viruses successfully exchanged genomes and that their HA genes are homologous.

Data Interpretation

It was concluded that in order to produce a highly virulent strain, the human strain must be phylogenetically similar to the avian strain. This can be observed with the PR8 strains, as most reassortants were able to provide similar-sized plaques as their parent viruses (no selection). All the reassortants were sequenced to find that they contained the human M genes of PR8. The less homologous the parent viruses, the less compatible the avian and human HA. Referring to table 3 with the Nanchang/95 strain, none of the reassortants were able to produce viable plaques, indicating poor cooperation. By successfully

Table 3: *Plaque yields (PFU) and maximum plaque sizes after single or double infection of MDCK cells with the human Nanchang and avain or swine influenza A viruses.*

Virus strain(s)	PFU (maximum plaque diam [mm])	
	No selection	Selection[a]
A/Nanchang/933/95 (H3N2)[b]	6×10^7 (4)	$<10^2$
A/Oystercatcher/Germany/87 (H1N1)	6×10^5 (2)	$<10^3$
A/Oystercatcher/Germany/87, Nanchang	2×10^6 (3)	$<10^3$
A/Swine/Germany/81 (H1N1)	1×10^8 (4)	$<10^3$
A/Swine/Germany/81, Nanchang	4×10^7 (4)	1×10^5 (0.3)
A/Mallard/Potsdam/178-4/83 (H2N2)	3×10^8 (6)	$<10^4$
A/Mallard/Potsdam/178-4/83, Nanchang	2×10^8 (6)	1×10^6 (2.5)
A/Duck/Ukraine/63 (H3N8)[c]	2×10^8 (6)	$<10^4$
A/Duck/Ukraine/63, Nanchang	8×10^7 (6)	4×10^6 (3)
A/Duck/Hong Kong/Y264/97 (H4N8)	2×10^7 (4)	$<10^4$
A/Duck/Hong Kong/Y264/97, Nanchang	1×10^7 (4)	1×10^5 (0.6)
A/Duck/Singapore/3/97 (H5N3)	6×10^7 (3)	$<10^3$
A/Duck/Singapore/3/97, Nanchang	6×10^7 (3)	4×10^4 (0.4)
A/Gray Teal/Australia/1/79 (H7N8)	8×10^6 (3)	$<10^3$
A/Gray Teal/Australia/1/79, Nanchang	8×10^6 (3)	1×10^4 (0.4)
A/Chick/Germany N/49 (H10N7)	6×10^7 (4)	$<10^4$
A/Chick/Germany N/49, Nanchang	4×10^7 (4)	1×10^5 (0.2)
A/Duck/Hong Kong/P50/97 (H11N9)	2×10^7 (3)	$<10^3$
A/Duck/Hong Kong/P50/97, Nanchang	1×10^7 (3)	3×10^4 (0.3)

[a] Anti-H3 antiserum (1:100 dilution in PBS) was used to select against human HA, and amantadine (4 μg/ml in the agar overlay) was used to select against avian and swine M genes.

[b] An amantadine-resistant Nanchang variant was used.

[c] The anti-H3 antiserum used did not neutralize the A/Duck/Uktraine/63 (H3N8) virus.

Table 3. Table from Scholtissek et al. (2002) to illustrate plaque yields and diameters for human Nanchang strain and avian influenza A. From *Cooperation between the Hemagglutinin of Avian Viruses and the Matrix Protein of Human Influenza A Viruses Christoph Scholtissek, Jürgen Stech, Scott Krauss, and Robert G. Webster J. Virol. February 2002 76:1781-1786; doi:10.1128/JVI.76.4.1781-1786.2002*

Figure 2: *Host and lineage origins for the gene segments of the 2009 A(H1N1) virus: PB2, PB1, PA, HA, NP, NA, M, NS. Color of gene segment in circle indicates host.*

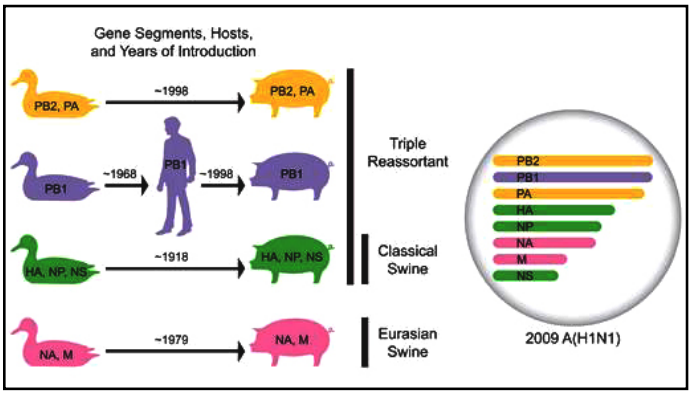

Figure 2. Host and lineage origins for the gene segments of the 2009 A(H1N1) virus: PB2, PB1, PA, HA, NP, NA, M, NS. Colour of the gene segment indicates the host. From *R. J. Garten, C. T. Davis et. al. SCIENCE. 325, 5937 (2009). Reprinted with permission from the American Association for the Advancement of Science (AAAS).*

forming a stable virus population within a mammalian host, the avian influenza virus forms a stable lineage. It increases the chances of surpassing the species barrier that prevents it from easily infecting humans. This finding is illustrated in Table 2, where only a few avian and avian-like swine strains were successful in recombination resulting in reasonably-sized plaques.

Errors

Although experiments were conducted twice to ensure that the data were reproducible, unexpected results occurred. For example, the evolution of plaques to become amantadine-sensitive was possible due to the lack of amantadine present and the nature of heterozygotic M-genes which determine the virus' resistance. Another explanation would be the chance of spontaneous mutation where a rare amantadine-resistant variant of the avian influenza viruses were to form.[1] The experimental model required human influenza viruses to be (Am+) and therefore, it would have skewed the survival rate of the cells.

Critical Analysis of Experimental Procedure

It is interesting that the Scholtissek et al. model assumes that avian HA and human M genes are the most relevant in determining cooperation between parent viruses. The influenza virion contains 8 main segments of viral RNA, two of which are HA and M. Recent studies on the origin of previous pandemic strains imply that 1918 A(H1N1), 1957 A(H2N2) and 1968 A(H3N2) were products of complex reassortment.[3-4] For instance, the 2009 A(H1N1) pandemic is believed to have been caused by the recombinant of at least three different swine viruses that were stable and circulating in Eurasia and America[4,6] (Figure 2), and not a simple reassortment between an avian and a human strain. Amongst the parent viruses, one of the Eurasian swine viruses' neuraminidase (NA) and M genes derived from a wholly-avian influenza virus.[4] This interaction is similar to the second set of experiments where the avian-like swine viruses were successfully reassorted with the Sing/57 (H2N2) strain, except with different genes. This implies that pandemic-planning should not focus on genetic recombination, but must consider reservoirs and how easily the virus may infect and accumulate within the host.

FUTURE DIRECTIONS

Although the chances of an influenza A pandemic circulating with HA subtypes other than 1, 2, and 3 is not possible, it is important to conduct further research in pandemic planning to prepare for future outbreaks. It takes series of multiple complex reassortments between many different stable circulating strains to form a potentially highly pathogenic novel strain. Further research needs to be done on influenza A reservoirs. Examples include swine and Eurasian swine as they have both "avian" type and "human" type HA receptors, enabling a low species barrier for mixing and distribution of different influenza A strains. Future research will be effective in seeking potential causes and virulence in pandemics.

ABBREVIATIONS

HA	Hemagglutinin
M	Matrix
TCPK	Tosylsulfonyl Phenylalanyl Chloromethyl Ketone-treated Trypsin
PB2	Polymerase Basic 2
PA	Polymerase Acidic
PBS	Phosphate-Buffered Saline
AM+	Amantadine-Resistant
AM-	Amantadine-Sensitive
MDCK	Madin-Darby Canine Kidney Cells
NA	Neuraminidase
PB1	Polymerase Basic 1
NP	Nucleoprotein
NS	Nonstructural Gene

KEY WORDS *Influenza; Pandemic; Hemagglutinin; Matrix; Reassortant*

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